Immunity, Vol. 8, 703–711, June, 1998, Copyright ©1998 by Cell Press

# Cabin 1, A Negative Regulator for Calcineurin Signaling in T Lymphocytes

**Luo Sun,* Hong-Duk Youn,* Christine Loh,*§ Melissa Stolow,† Weiwu He,† and Jun O. Liu*‡**

*Center for Cancer Research
and Departments of Biology and Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

†OriGene Technologies Incorporated
Rockville, Maryland 20850

## Summary

**Calcineurin plays a pivotal role in the T cell receptor (TCR)–mediated signal transduction pathway and serves as a common target for the immunosuppressants FK506 and cyclosporin A. We report the identification of a novel endogenous calcineurin binding protein named Cabin 1 that inhibits calcineurin-mediated signal transduction. The interaction between Cabin 1 and calcineurin is dependent on PKC activation. Overexpression of Cabin 1 or its N-terminal truncation mutants inhibits the transcriptional activation of calcineurin-responsive elements in the interleukin-2 promoter and blocks dephosphorylation of NF-AT upon T cell activation. These results suggest a negative regulatory role for Cabin 1 in calcineurin signaling and provide a possible mechanism of feedback inhibition of TCR signaling through cross-talk between protein kinases and calcineurin.**

## Introduction

Calcineurin (also known as PP-2B), a calcium and calmodulin-dependent protein serine/threonine phosphatase, is a rate-limiting enzyme in T cell receptor (TCR)–mediated signal transduction leading to the transcriptional activation of cytokines such as IL-2 (Crabtree and Clipstone, 1994). The role of calcineurin in TCR signaling was first revealed when it was identified as the common target for the immunosuppressive drugs cyclosporin A (CsA) and FK506 (Friedman and Weissman, 1991; Liu et al., 1991). Upon entering T cells, CsA binds to cyclophilin (Handschumacher et al., 1984), and FK506 binds to FKBP (Harding et al., 1989; Siekierka et al., 1989). The cyclophilin–CsA and FKBP–FK506 complexes independently associate with calcineurin and inhibit its protein phosphatase activity (Friedman and Weissman, 1991; Liu et al., 1991). Substantial pharmacological and cell biological evidence now exists supporting an essential role of calcineurin in the TCR signal transduction pathway (Clipstone and Crabtree, 1992; Fruman et al., 1992; Liu et al., 1992; O'Keefe et al., 1992; Nelson et al., 1993).

‡To whom correspondence should be addressed (e-mail: junliu@mit.edu).

§Present address: ICOS Corporation, 22021 20th Avenue S.E., Bothell, WA 98021.

Calcineurin is known to modulate the activity of several transcription factors that bind to the IL-2 promoter, including NF-AT, NF-κB, and AP-1 (Emmel et al., 1989; Frantz et al., 1994; Su et al., 1994). Among these transcription factors, calcineurin has been shown to dephosphorylate the cytoplasmic subunit of NF-AT, allowing it to translocate into the nucleus to activate transcription (Flanagan et al., 1991; Shaw et al., 1995; Loh et al., 1996; Timmerman et al., 1996). Recent studies have shown that activated calcineurin comigrates with the cytoplasmic subunit of NF-AT into the nucleus, where it may be required in sustaining the activation of NF-AT (Shibasaki et al., 1996). In addition to the cytoplasmic subunit of NF-AT, calcineurin has been shown to dephosphorylate the transcription factor Elk-1, down-regulating its transcriptional activity (Sugimoto et al., 1997). Calcineurin has also been reported to bind several other cellular proteins, including the protein kinase A anchoring protein (AKAP79) (Coghlan et al., 1995; Klauck et al., 1996) and the $IP_3$ and ryanodine receptors (Cameron et al., 1995). To what extent these calcineurin-associated proteins are involved in TCR signaling remains to be determined.

Calcineurin belongs to a superfamily of protein serine/threonine phosphatases (Klee et al., 1988; Cohen, 1989; Shenolikar, 1994). Unlike other members of this superfamily, however, the activity of calcineurin is controlled by intracellular calcium concentrations. This is made possible by the unique structure of the enzyme. Calcineurin consists of two subunits, a catalytic subunit (A) and a regulatory subunit (B). The A subunit contains several distinct structural domains, including a catalytic domain, a B subunit binding domain, a calmodulin binding domain, and an autoinhibitory domain (Figure 1A). The calmodulin binding domain and the autoinhibitory domain together form the calcium-sensing on–off switch. At low calcium concentrations in quiescent T cells, the autoinhibitory domain of calcineurin binds to the catalytic domain, keeping calcineurin inactive (Hashimoto et al., 1990). During T cell activation, the increase in intracellular calcium concentration leads to activation of calmodulin, which interacts with the calmodulin binding domain of calcineurin A, releasing the autoinhibitory domain of calcineurin from its active site and activating its phosphatase activity. Proteolytic removal of the autoinhibitory domain and a point mutation within the autoinhibitory domain have been shown to give rise to a constitutively active form of calcineurin (Hubbard and Klee, 1989; Fruman et al., 1995).

Although much is known about how calcineurin is activated, little is known about how it is down-regulated following T cell activation. We report herein the identification of a novel calcineurin binding protein named Cabin 1, which binds to and inhibits the phosphatase activity of calcineurin. The interaction between Cabin 1 and calcineurin is dependent on both PKC and calcium signals. We show that Cabin 1 is a phosphoprotein, and PKC activation leads to its hyperphosphorylation. Expression of Cabin 1 or its C-terminal fragments blocks

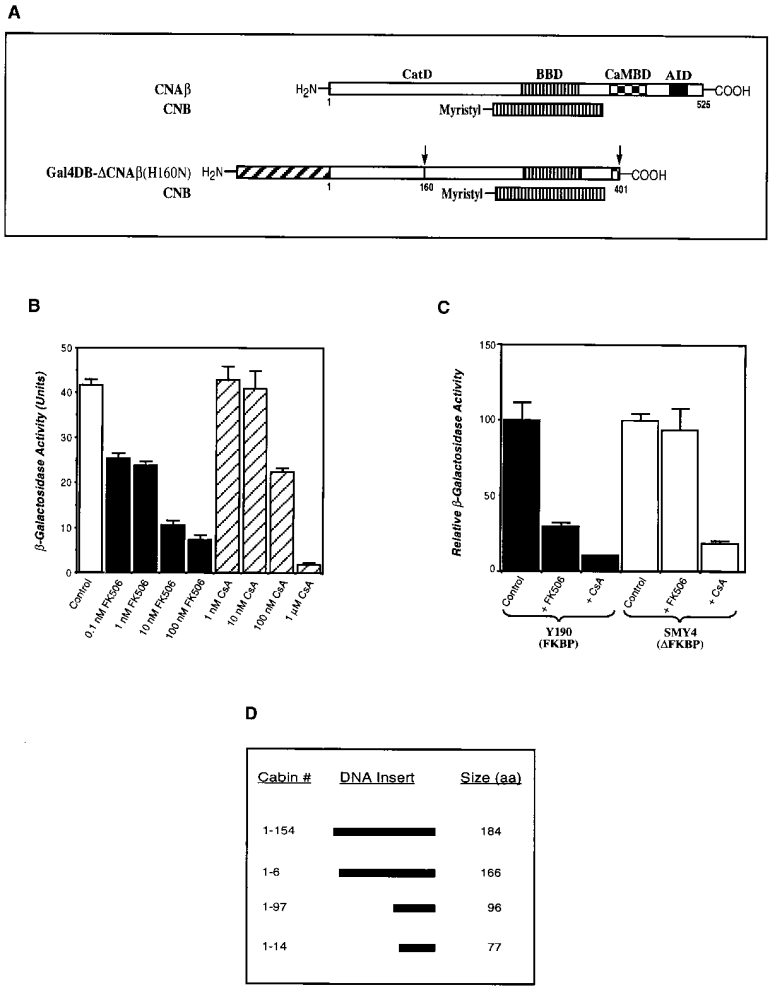


Figure 1. Yeast Two-Hybrid Screen for Calcineurin-Interacting Proteins

(A) Schematic diagrams of wild-type calcineurin and the H160N mutant used as bait. Abbreviations: CN, calcineurin; CatD, catalytic domain; BBD, CNB binding domain; CaMBD, calmodulin binding domain; AID, autoinhibitory domain. Arrows indicate the sites of mutation and truncation, respectively, in the calcineurin bait.

(B) Sensitivity of interaction between Gal4-ΔCNβ2(H160N) and Cabin 1-14 (see Figures 1D and 2A) to FK506 and CsA. The data for the remaining three cabin clones are similar to that for Cabin 1-14.

(C) Sensitivity of interaction between Gal4-DCNβ2(H160N) and Cabin 1-14 to FK506 is dependent on the presence of endogenous FKBP12. The concentrations of FK506 and CsA used were 100 nM and 1 μM, respectively.

(D) Schematic representation of overlapping clones of Cabin 1 identified from the yeast two-hybrid screen. All four clones end with the stop codon of the full-length cDNA (see Figure 2A).

transcriptional activation of an IL-2 reporter gene in response to PMA and ionomycin and inhibits the dephosphorylation of NF-AT in vivo. Cabin 1 may thus serve as an endogenous inhibitor for calcineurin and play a negative regulatory role in calcineurin signaling.

## Results

### Identification of Cabin 1 as a Calcineurin Binding Protein by Yeast Two-Hybrid Screen

To identify new calcineurin substrates and interacting proteins, we employed a catalytically inactive calcineurin mutant as an affinity probe, reasoning that it would associate with substrates more stably than the wild-type enzyme, as has been demonstrated for protein tyrosine phosphatases (Sun et al., 1993; Furukawa, 1995). Through site-directed mutagenesis of residues at the putative active site of calcineurin, we previously found a number of mutants that are catalytically inactive but retain structural integrity as judged by their interactions with the FKBP–FK506 complex (Mondragon et al., 1997). We created a H160N mutant of the β2 isoform of calcineurin, the predominant isoform in T lymphocytes (Jiang et al., 1997). When expressed in T cells, this mutant was found to be dominant negative, blocking PMA- and ionomycin-stimulated IL-2 promoter activation by over 60% (data not shown), in agreement with the previous finding that expression of a similar mutant (H160Q) inhibited nuclear translocation of NF-AT4 (Shibasaki et al., 1996), a known calcineurin substrate (Jain et al., 1993; Wesselborg et al., 1996; Beals et al., 1997). We therefore chose the H160N mutant of the β2 isoform of calcineurin as the bait in the yeast two-hybrid system (Fields and Song, 1989; Durfee et al., 1993) to identify its substrates and binding proteins. To alleviate the dependence of full-length calcineurin on calcium in yeast, the C-terminal autoinhibitory domain and most of the calmodulin binding domain were removed from the catalytic subunit in the calcineurin bait (Figure 1A). To validate this strategy, we constructed a fish plasmid encoding a fusion protein between the N-terminal domain of NF-AT1 and the Gal4 activation domain and tested whether the fusion protein interacted with the calcineurin bait in the two-hybrid system. Significant activation of the *lacZ* reporter gene was observed in yeast expressing the calcineurin bait and NF-AT fish fusion proteins (data not shown), indicating that the use of the

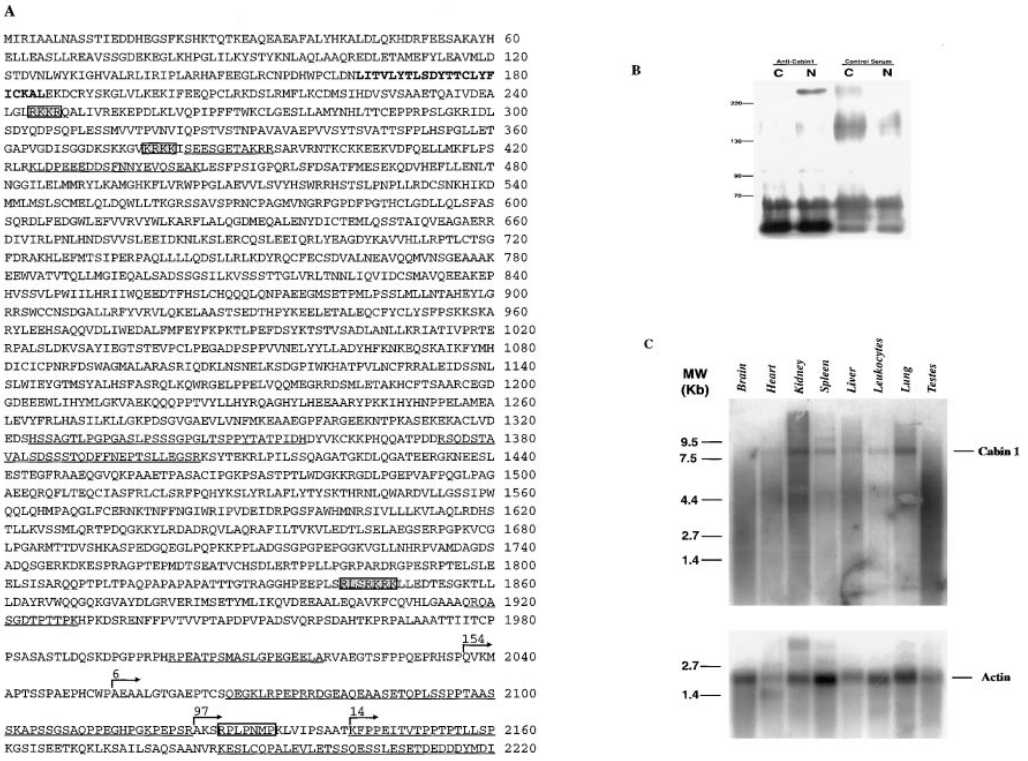


Figure 2. Amino Acid Sequence and Tissue and Subcellular Distribution of Cabin 1

(A) Amino acid sequence of the putative full-length Cabin 1. Highlighted are a putative coiled-coil domain (bold); putative nuclear localization sequences (boxed and shaded); PEST sequences (underlined); and a putative SH3 binding site (boxed). The N termini of the four Cabin 1 clones isolated by the yeast two-hybrid screen are highlighted.

(B) Cabin 1 is a nuclear protein. Jurkat T cell lysates are fractionated into cytostolic (C) and nuclear (N) fractions, and Cabin 1 was immunoprecipitated using anti–Cabin 1 antibodies. After SDS-PAGE, the immunoprecipitated Cabin 1 was blotted onto a nitrocellulose membrane and probed with anti–Cabin 1 antibodies or control serum.

(C) Northern blot showing Cabin 1 mRNA is widely expressed in different tissues.

catalytically inactive mutant of calcineurin may be fruitful for identifying substrates and interacting proteins.

Upon screening 1.6 million independent transformants from a mouse T cell cDNA library (Elledge et al., 1991), we identified six clones that exhibited significant activation of the *lacZ* reporter gene. To determine the specificity of these clones for calcineurin, we performed the yeast two-hybrid assay in the presence of FK506 and CsA, which inhibit binding of substrates to calcineurin (Liu et al., 1991). As shown in Figure 1B, the interaction between one of the clones and the mutant calcineurin bait can be inhibited by FK506 and CsA in a dose-dependent manner. The remaining five clones exhibited similar sensitivity to FK506 and CsA (data not shown). FK506 was found to be over 10-fold more potent than CsA, in agreement with the relative potencies of these drugs in T cells (Kino et al., 1987). To further assess the specificity of the interaction, the two-hybrid assay was performed in the yeast strain SMY4 that lacks FKBP12 (Cardenas et al., 1994). In SMY4, the interaction became resistant to FK506 while remaining sensitive to CsA, indicating that the sensitivity to FK506 is mediated by FKBP12 and is specific for calcineurin (Figure 1C). Sequence analysis revealed that four of the six clones encode overlapping cDNAs of the same gene (Figure 1D); the corresponding calcineurin binding protein was named Cabin 1.

### Cabin 1 Is a Novel Nuclear Protein with Multiple Structural Motifs

Using a combination of EST database searching and PCR cloning, the full-length human Cabin 1 was cloned to give a complete open reading frame encoding 2220 amino acids (Figure 2A). A database search revealed that Cabin 1 is a novel protein. Amino acid analysis of the conceptually translated sequence revealed a leucine zipper, a putative SH3 binding site, and three putative nuclear localization sequences in Cabin 1 (Figure 2A). In addition, Cabin 1 contains several PEST sequences that have been proposed to target a wide variety of cellular proteins for degradation, including nuclear protein kinase C, IκBα, Fos, p53, and G1 cyclins (Rechsteiner and Rogers, 1996) (Figure 2A). There are a number of consensus phosphorylation sites for various kinases, including PKA, PKC, and MAP kinases (data not shown). Immunoprecipitation of Cabin 1 from fractionated cell lysates followed by Western blot using polyclonal anti–Cabin 1 antibodies confirmed that Cabin 1 is a nuclear protein (Figure 2B). Cabin 1 remains in the nucleus upon treatment of Jurkat T cells with PMA and ionomycin (data not shown). Northern analysis indicated that Cabin 1 is widely expressed in a variety of tissues, including the spleen and leukocytes (Figure 2C).

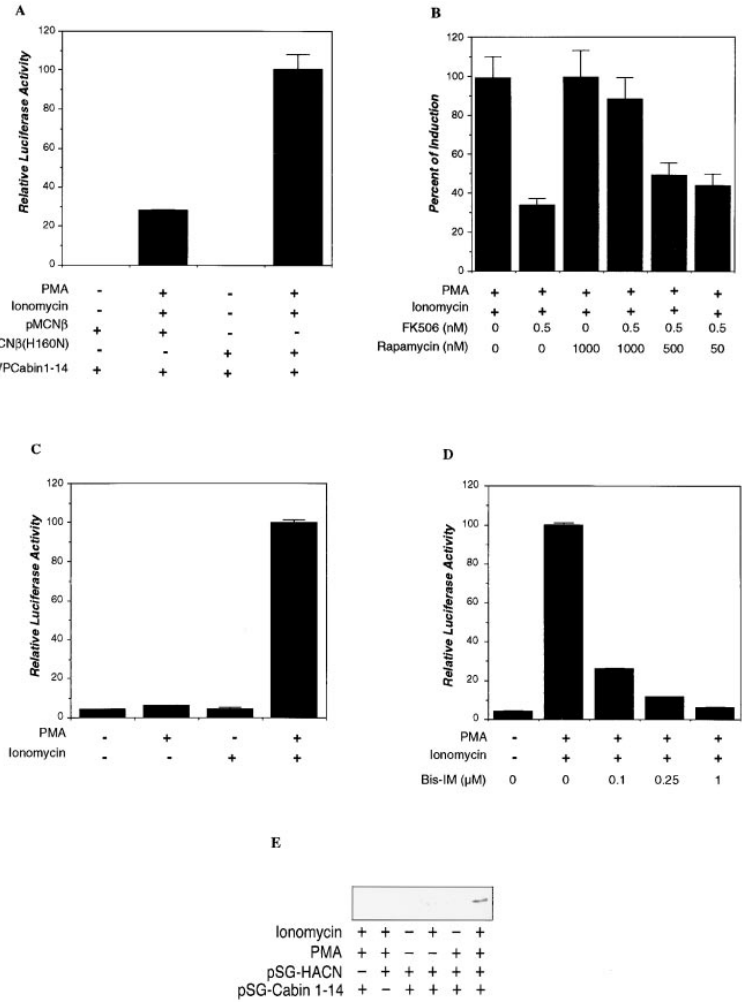


Figure 3. Cabin 1 Specifically Interacts with Wild-Type and Mutant Calcineurin in Jurkat T Cells in a PMA- and Ionomycin-Dependent Fashion

(A) Interaction between Cabin 1-14 and full-length wild-type or H160N calcineurin β2 in Jurkat T cells can be detected by a mammalian two-hybrid assay.

(B) The interaction between Cabin 1-14 and full-length calcineurin β2(H160N) is sensitive to FK506, and the inhibition by FK506 is antagonized by an excess of rapamycin.

(C) The interaction between Cabin 1-14 and full-length calcineurin β2(H160N) requires stimulation by both PMA and ionomycin in the mammalian two-hybrid assay.

(D) The interaction between Cabin 1-14 and full-length calcineurin β2(H160N) is sensitive to the PKC inhibitor bisindolylmaleimide.

(E) Coimmunoprecipitation of Cabin 1-14 and wild-type calcineurin β2 is dependent on both PKC and calcium stimulation.

### Interaction between Cabin 1 and Calcineurin Is Dependent on Both PKC and Calcium Signals

To verify the interaction between Cabin 1 and calcineurin in T cells, we established a mammalian two-hybrid system in Jurkat T cells by fusing calcineurin with the Gal4 DNA binding domain and Cabin 1-14 with the VP16 activation domain (Hsu et al., 1994). One advantage of this system is that it allows for the use of various forms of full-length calcineurin that can be activated by calcium signal. When stimulated with PMA and ionomycin, the full-length calcineurin β2(H160N) mutant showed strong interaction with Cabin 1-14 (Figure 3A). While the wild-type calcineurin also interacts with Cabin 1-14, the interaction is several-fold weaker than that for the mutant enzyme. The alteration of the active site structure in the mutant appeared to have caused an increase in affinity of calcineurin for Cabin 1. Similar to the observation made in the yeast two-hybrid assay (Figure 1B), the interaction between the full-length calcineurin β2(H160N) mutant and Cabin 1-14 is sensitive to FK506 (Figure 3B) and CsA (data not shown). Addition of rapamycin, an immunosuppressant that binds FKBP12 but does not inhibit TCR-mediated signaling or calcineurin (Sehgal et al., 1975; Bierer et al., 1990; Liu et al., 1991), reverts the inhibitory effect of FK506 in a dose-dependent manner, indicating that the interaction between Cabin 1 and calcineurin is highly specific (Figure 3B).

In the mammalian two-hybrid system, the interaction between full-length calcineurin and Cabin 1-14 requires ionomycin, which is consistent with the activation of full-length calcineurin by calcium and calmodulin. Unexpectedly, this interaction also requires PMA (Figure 3C). Since PMA is known to activate PKC, we examined the effect of a PKC-specific inhibitor, bisindolylmaleimide (Toullec et al., 1991), on the PMA/ionomycin-stimulated interaction between Cabin 1-14 and calcineurin. Bisindolylmaleimide inhibited the interaction between calcineurin and Cabin 1-14 in a dose-dependent manner, suggesting that the requirement for PMA is mediated through PKC activation (Figure 3D). In addition to the mammalian two-hybrid system, the interaction of Cabin 1-14 and the wild-type full-length calcineurin was also confirmed by coimmunoprecipitation (Figure 3E). In agreement with the observations made in the mammalian two-hybrid system, Cabin 1-14 and HA-tagged calcineurin (HACN) coprecipitate only upon stimulation of Jurkat T cells with both PMA and ionomycin (Figure 3E). Thus, Cabin 1 interacts with calcineurin in T cells in a calcium- and PKC-dependent manner.

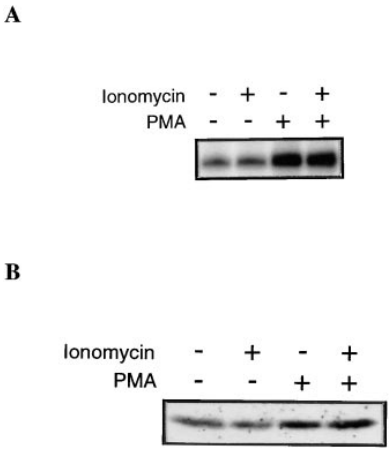


Figure 4. Activation of PKC Induces Hyperphosphorylation of Cabin 1-14

(A) $^{32}P$ labeling of Cabin 1-14 in the presence or absence of PMA and ionomycin in Jurkat T cells.

(B) Western blot analysis of transfected Cabin 1-14 in the presence or absence of PMA and ionomycin.

### PKC Activation Leads to Hyperphosphorylation of Cabin 1

The dual requirement of Cabin 1–calcineurin interaction for calcium and PKC signals raised the question of whether PKC modulates this interaction by acting on calcineurin or Cabin 1. Calcineurin is not known to be subject to regulation by phosphorylation. The presence of multiple putative kinase phosphorylation sites in Cabin 1 suggested that Cabin 1 may be subject to PKC-stimulated phosphorylation. To test this possibility and to determine whether the phosphorylation state of Cabin 1 is regulated during T cell activation, we labeled Jurkat T cells transfected with an expression plasmid for Cabin 1-14 with $^{32}P$-inorganic phosphate in the presence of PMA, ionomycin, or both. As shown in Figure 4A, Cabin 1-14 is phosphorylated in unstimulated Jurkat T cells. Treatment with PMA leads to hyperphosphorylation of Cabin 1-14, as evidenced by a higher level of $^{32}P$ incorporation and a shift in gel mobility. In contrast, the expression level of Cabin 1-14 was only slightly affected by PMA (Figure 4B). Treatment with ionomycin did not affect the phosphorylation state of Cabin 1-14, suggesting that calcium–calcineurin signaling does not dephosphorylate Cabin 1-14. Consistent with those observations, immunoprecipitated Cabin 1-14 is not dephosphorylated by recombinant calcineurin in vitro (data not shown). These results also suggest that the dependence of calcineurin–Cabin 1 interaction on PKC is likely mediated by the PKC-induced hyperphosphorylation of Cabin 1.

### Expression of Cabin 1 or Its C-Terminal Fragments Blocks IL-2 Promoter Activation in Response to PMA and Ionomycin

Given that Cabin 1 binds to calcineurin in T cells, we determined whether expression of Cabin 1 or the C-terminal fragment Cabin 1-14 affects calcineurin signaling upon T cell activation. Thus, Cabin 1-14 or the empty vector were cotransfected into Jurkat T cells with a luciferase reporter gene under the control of the IL-2 promoter. Upon treatment with PMA and ionomycin, the IL-2 reporter gene is activated (Figure 5A). This activation is inhibited by FK506 but is unaffected by cotransfection of the empty vector. Coexpression of Cabin 1-14, however, potently inhibited the activation of the IL-2 reporter gene (Figure 5A). Similar to Cabin 1-14, the full-length Cabin 1 is also capable of inhibiting the IL-2 reporter gene activation in a dose-dependent manner, albeit with lower potency, suggesting that inhibition of calcineurin signaling is an intrinsic activity of Cabin 1 (Figure 5A). Furthermore, as Cabin 1 is localized in the nucleus (Figure 2C), the inhibitory effect of full-length Cabin 1 on the IL-2 reporter gene also implicates that the presence of activated calcineurin in the nucleus is required for IL-2 reporter gene activation in response to PMA and ionomycin. To further identify the specific elements in the IL-2 promoter that are sensitive to the inhibition by Cabin 1-14, we tested reporter genes under the control of multimerized AP-1, ARRE (Oct1/AP-1), NF-AT, and NF-κB binding sites derived from the IL-2 promoter (Figure 5B). All four reporter genes are inhibited by coexpression of Cabin 1-14, suggesting that the inhibitory effect of Cabin 1-14 may result from inhibition of calcineurin since these reporters are known to be sensitive to FK506 and CsA.

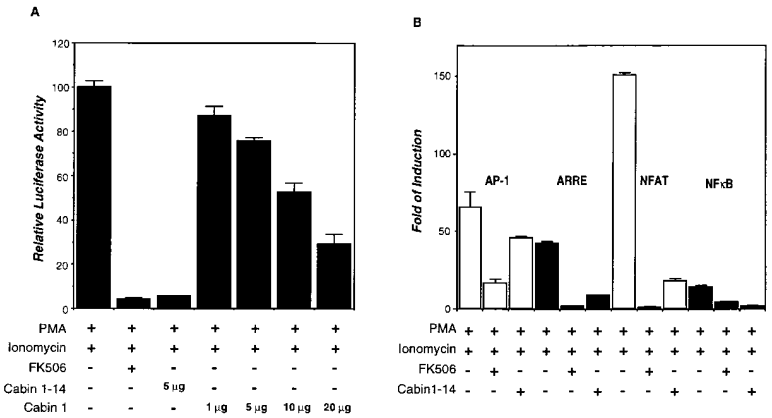


Figure 5. Dominant Inhibitory Effect of Overexpression of Cabin 1-14 on the Transcriptional Activation of Different Calcineurin-Responsive Elements in the Interleukin-2 Promoter and on the Dephosphorylation of NF-AT in Response to PMA/Ionomycin Stimulation

(A) Both Cabin 1-14 and Cabin 1 inhibit the activation of IL-2/Luc reporter gene.

(B) Cabin 1-14 inhibits the activation of AP1/Luc, ARRE/Luc, NF-AT/Luc, and NF-κB/Luc reporter genes.

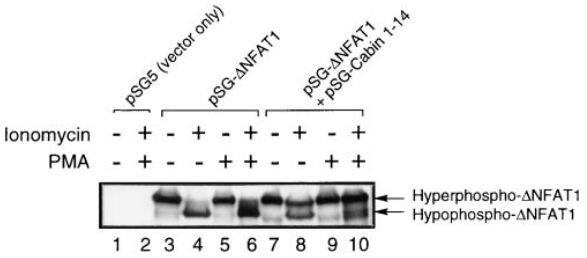


Figure 6. Dephosphorylation of ΔNF-AT1 in Response to Ionomycin/PMA Treatment Is Inhibited by Cabin 1-14

Jurkat T cells were transfected with various plasmids and allowed to recover for 48 hr. Cells were then stimulated with PMA, ionomycin, or both for 1 hr. Whole-cell lysates were prepared, subjected to 8% SDS-PAGE, transferred to nitrocellulose and probed with anti-HA epitope monoclonal antibody.

### Cabin 1-14 Inhibits Dephosphorylation of NF-AT by Calcineurin In Vivo

To determine if the inhibition of IL-2 promoter by Cabin 1-14 is mediated through the inhibition of calcineurin phosphatase activity in vivo, we examined the effect of Cabin 1-14 on the calcineurin-mediated dephosphorylation of the N-terminal fragment of NF-AT1 (ΔNF-AT1) upon treatment with ionomycin and PMA. Overexpression of Cabin 1-14 inhibited the dephosphorylation of ΔNF-AT1 upon treatment with ionomycin in the presence or absence of PMA (Figure 6). It is noteworthy, however, that Cabin 1-14 inhibited ΔNF-AT1 dephosphorylation to a larger extent in the presence of PMA than in its absence (Figure 6, lane 8 versus lane 10). These observations are consistent with the PKC-dependence of the mammalian two-hybrid interaction between Cabin 1-14 and calcineurin (Figure 3D) and suggest that normally phosphorylated Cabin 1-14 can interact with calcineurin with low affinity, and hyperphosphorylation of Cabin 1-14 upon PKC activation leads to higher affinity for calcineurin. They also support the notion that hyperphosphorylated Cabin 1-14 binds to, and inhibits the protein phosphatase activity of, calcineurin in vivo.

## Discussion

We have identified a novel calcineurin binding protein, Cabin 1, that is capable of binding to and inhibiting the phosphatase activity of calcineurin. The interaction between Cabin 1 and calcineurin was confirmed both in a mammalian two-hybrid system and by coimmunoprecipitation. Overexpression of Cabin 1 or its C-terminal calcineurin-binding fragments inhibits calcineurin-mediated IL-2 reporter gene activation and dephosphorylation of NF-AT, suggesting that Cabin 1 may serve as a negative regulator of TCR signaling via inhibition of calcineurin.

We showed that Cabin 1 is a nuclear protein. But calcineurin is known as a cytosolic enzyme, raising the question of whether Cabin 1 can form a physiologically relevant complex with calcineurin. Recently, it was shown that calcineurin migrates into the nucleus via a complex with NF-AT upon T cell activation (Shibasaki et al., 1996). It is possible that Cabin 1 is responsible for regulating the function of calcineurin after its translocation into the nucleus. This is consistent with our observations that Cabin 1 is specific for the activated form of calcineurin. The fact that nuclearly localized full-length Cabin 1 is capable of blocking calcineurin-mediated IL-2 reporter gene activation (Figure 5A) further implicates a role for calcineurin in the nucleus during TCR signaling.

The interaction between Cabin 1 and calcineurin requires both calcium and PKC signals as demonstrated by the mammalian two-hybrid system and coimmunoprecipitation of the two proteins. The calcium signal is required for activation of calcineurin, suggesting that Cabin 1 only interacts with activated calcineurin. Activation of PKC leads to hyperphosphorylation of Cabin 1 that appears to be required for its high-affinity interaction with calcineurin. We have not shown that calcineurin and Cabin 1 interact upon TCR-induced phosphorylation of Cabin 1 using endogenous proteins due, in large part, to the low levels of Cabin 1 and calcineurin expressed in T cells. It also remains to be determined whether the PKC-dependent hyperphosphorylation of Cabin 1 is direct or indirect. Our preliminary data suggest that the hyperphosphorylation of Cabin 1 mediated by PKC is an indirect result of the activation of other kinases by PKC (L. S., H.-D. Y., and J. O. L., unpublished data). The existence of a large number of serine and threonine residues and multiple consensus phosphorylation sites in Cabin 1 suggests that it may be subject to regulation by multiple kinases. The identification of the kinases involved in the phosphorylation of Cabin 1 and the corresponding phosphorylation sites mediating its interaction with calcineurin will be important for a full understanding of the cross-talk between calcineurin and kinases during T cell activation.

In contrast to the mammalian cells, the interaction between Cabin 1 and calcineurin in yeast is not dependent on PKC. Although whether Cabin 1 is phosphorylated in yeast is not known, it is possible that yeast contains kinases that have overlapping specificity as the mammalian kinases that phosphorylate Cabin 1 given that calcineurin is also present in yeast. It is also possible that the high level of overexpressed Cabin 1 C-terminal fragments may compensate for their low affinity in the absence of phosphorylation, rendering their interactions with the calcineurin bait strong enough to give sufficient reporter gene activity.

Two alternative models can account for the function of Cabin 1 during T cell activation. A phosphoprotein pseudosubstrate inhibitor, PP-1I, is known for another serine/threonine phosphatase, PP-1, that belongs to the same superfamily of enzymes as calcineurin (Aitken et al., 1982). PP-1I does not bind to PP-1 unless it is phosphorylated on a threonine residue. Like Cabin 1, PP-1I also contains PEST sequences (Rechsteiner and Rogers, 1996). It is possible that hyperphosphorylated Cabin 1 binds to and inhibits the phosphatase activity of calcineurin in a manner analogous to that of PP-1I, serving as an endogenous inhibitor for calcineurin. Alternatively, Cabin 1 may serve as a nuclear anchoring protein for calcineurin during T cell activation. This role is precedented by the A kinase anchoring protein (AKAP79) that serves as a scaffold protein to anchor calcineurin, PKA, and PKC to the same subcellular locations in neuronal

cells (Coghlan et al., 1995; Klauck et al., 1996). Similar to Cabin 1, the calcineurin binding domain of AKAP79 can also inhibit the phosphatase activity of calcineurin. Given that Cabin 1 contains multiple structural motifs in addition to the calcineurin binding domain, it has the potential to bind simultaneously to multiple proteins along with calcineurin. The fact that expression of full-length Cabin 1 inhibits IL-2 reporter gene activation in response to PMA and ionomycin is more consistent with the first model in which Cabin 1 plays a negative regulatory role in calcineurin signaling (Figure 5A).

The TCR signaling pathway is known to be down-regulated in the course of T cell activation (Gardner, 1989). Activation of PKC has been previously shown to antagonize calcium signaling (Tordai et al., 1989). Through inhibition of calcineurin in a PKC-dependent fashion, Cabin 1 may serve in an internal negative feedback loop to inhibit this pathway. Thus, Cabin 1 and calcineurin are localized to the nucleus and cytosol, respectively, in quiescent T cells. Upon T cell activation, a subpopulation of calcineurin translocates into the nucleus to maintain the transcriptional activity of NF-AT and possibly other factors (Shibasaki et al., 1996). Activation of PKC and other kinases leads to the hyperphosphorylation of Cabin 1, converting it into a high-affinity inhibitor for calcineurin, thereby attenuating TCR signaling. It is noteworthy that the calcineurin binding domain of Cabin 1 occupies only a small portion of the entire protein. The presence of a leucine zipper motif, an SH3 binding site, and a number of putative phosphorylation sites in Cabin 1 suggests that it is likely to be a multifunctional protein, capable of mediating additional signaling connections and playing other roles in T lymphocytes.

## Experimental Procedures

### Yeast Two-Hybrid Screen

Cabin 1 cDNA fragments were isolated by a yeast two-hybrid screen of a mouse T cell cDNA library (obtained from Dr. Steve Elledge). The bait plasmid was constructed by the insertion of a cDNA fragment encoding the first 410 amino acids of CNβ2 with a mutation (H160N) into the BamHI and Sal1 sites in pAS2 to give pAS-CNβ2m. Subsequently, an expression cassette of CNB under the control of yeast ADH promoter was inserted into a blunted SacI site of pAS-CNβ2m. The calcineurin bait plasmid was transformed into the yeast strain Y190, followed by transformation with the mouse T cell cDNA library (Elledge et al., 1991). The transformed yeast were selected on dropout plates ($His^-$, $Leu^-$, $Trp^-$) with 50 mM 3-amino-1,2,4-triazole. Positive colonies were picked 5 days after plating and streaked on X-Gal ($Leu^-$, $Trp^-$) plates. Colonies that turned blue overnight were further subjected to liquid β-gal assay to confirm the *lacZ* reporter gene expression. DNA from those colonies were retrieved and sequenced.

### Cloning of Cabin 1 cDNA

The longest mouse Cabin 1 cDNA fragment obtained from the yeast two-hybrid screen was used to search the EST database. Two overlapping cDNA fragments encoding a human Cabin 1 were identified. They were obtained from Dr. Takahiro Nagase, Kazusa DNA Research Institute, and Genome System Inc., respectively. Using a primer derived from these clones, a RACE PCR was performed with a human kidney cDNA library to obtain a 1.5 kb cDNA fragment corresponding to the remaining 5′ region of Cabin 1 cDNA. The sequences of these clones were confirmed, and the longest ORF encodes 2220 amino acids with a consensus Kozak initiation sequence preceded by stop codons.

### Nuclear Localization of Endogenous Cabin 1

Nuclear extract was prepared from Jurkat cells as previously described with slight modifications (Dignam et al., 1983). Exponentially growing Jurkat T cells were harvested and Dounce-homogenized in a homogenization buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, and 1 mM PMSF). Nuclei were precipitated by centrifugation at 1,000 × g for 10 min (the supernatant was taken as the cytosolic fraction), washed twice, and lysed in a lysis buffer (20 mM HEPES [pH 7.9] 0.1 M NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 1 mM PMSF, 1% Triton X-100, and 0.5% NP-40). Immunoprecipitation was carried out using both nuclear and cytosolic fractions with anti-Cabin 1 polyclonal antibodies. This is followed by immunoblotting with anti-Cabin 1 antibodies or a control serum.

### Northern Blot Analysis

A 4 kb fragment (EcoRI, BamHI) from the 5′ region of mouse Cabin 1 cDNA was labeled with [α-$^{32}$P]dCTP by random priming using the T7 Quick-Prime Kit (Pharmacia) according to the manufacturer's instructions. Radiolabeled Cabin 1 fragment was purified through NucTrap Probe Purification column (Stratagene) and used to probe a human multiple-tissue Northern blot (OriGene, Rockville, MD). The membrane was hybridized at 42°C for 20 hr and then washed at 65°C in 2 × SSC/0.1% SDS for 1 hr followed by another wash in 0.2 × SSC/0.1% SDS for 30 min and a final wash in 0.1 × SSC/0.1% SDS for 30 min.

### Coimmunoprecipitation of Cabin 1-14 and Calcineurin

Jurkat T cells transfected with pSG-HACNAβ2 and pSG-Cabin 1-14 were stimulated with PMA, ionomycin, or both for 3 hr. The cells were lysed in a lysis buffer (20 mM TrisHCl [pH 7.4], 0.1 M NaCl, 1% Triton X-100, 0.5% NP-40, and 0.5 mM PMSF). Cell lysates thus prepared were incubated with anti-Cabin 1 polyclonal antibodies and protein A/G agarose (Santa Cruz) for 2 hr, washed with lysis buffer, and boiled in SDS sample buffer. The samples were subjected to 10% SDS-PAGE, transferred to nitrocellulose that was probed with anti-HA monoclonal antibodies, and developed with ECL reagents (Amersham).

### Mammalian Two-Hybrid Assays for Interaction between Calcineurin and Cabin 1

The fusion plasmids for mammalian two-hybrid assays were constructed as follows. The cDNAs of calcineurinAβ2 wild-type and H160N mutant were fused to the GAL4 DB in the pM vector (Clontech) [pMCNβ2 and pMCNβ2(H160N)], and the cDNA of Cabin 1-14 was fused to the VP16 AD in the pVP16 vector (pVPCabin1-14). Jurkat T cells were transfected with 5 μg of pMCNβ2 or pMCNAβ2(H160N), 5 μg of pVPCabin1-14, 1 μg of pCMV-βGal, and 2 μg of pG5Luc, a luciferase reporter plasmid containing GAL4 promoter, by electroporation (960 μF, 250 V). After 48 hr, cells were stimulated with 10 nM PMA and 1 μM ionomycin, either alone or in combination, for 6 hr before luciferase activity was measured for each sample. Where applied, FK506, rapamycin, cyclosporin A, and bisindolylmaleimide were added to Jurkat cells 30 min prior to the stimulation.

### Reporter Gene Assays

The luciferase reporter gene assay reagents were obtained from Promega, and the assay was performed per manufacturer's instructions. The transient transfection was performed using electroporation. Jurkat cells ($1 \times 10^7$) were harvested, washed once in RPMI medium, and mixed with 2 μg of luciferase reporter plasmid and 0–5 μg of expression plasmid for Cabin 1-14 in 300 μl of RPMI in a sterile cuvette. An electric pulse (250 V, 960 mF) was applied (Bio-Rad Gene Pulser II). Where appropriate, the transfection efficiency was measured by cotransfecting 2 μg of CMV-driven β-galactosidase expression vector and measuring enzymatic β-galactosidase activity (Ausubel et al., 1994).

### Dephosphorylation of NF-AT by Calcineurin In Vivo

Jurkat T cells were transfected with expression vectors for Cabin 1-14 (pSGCabin 1-14) and for HA-epitope-tagged truncated NF-AT1(1–460) fused to green fluorescent protein [pSG-ΔNF-AT1

(1–460)] by electroporation as described above. After 48 hr, cells were treated with PMA and ionomycin, either alone or in combination, for another 1 hr. Whole-cell lysates were prepared and subjected to 8% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Schleicher and Schuell, NH), probed with anti-HA epitope monoclonal antibody, and developed with ECL reagents (Amersham).

**Acknowledgments**

We are grateful to Takahiro Nagase for the N-terminal human Cabin 1 cDNA clone (gene name KIAA 0330). We thank Steve Elledge for the mouse T cell cDNA library, Joe Heitman for the SMY4 yeast strain, Anjana Rao for an NF-ATp plasmid, Bin Su and Tieliang Deng for reporter plasmids, Jerry Crabtree for Tag Jurkat T cells, and Genhong Chen for advice on the yeast two-hybrid screen. We are grateful to Eric Griffith and Ben Turk for critically reading the manuscript. We thank Chris Armstrong for initiating yeast two-hybrid screens for cabins. This work was supported in part by the Chicago Community Trust, the Rita Allen Foundation, and the National Institutes of Health.

Received May 5, 1998; revised May 20, 1998.

**References**

Aitken, A., Bilham, T., and Cohen, P. (1982). Complete primary structure of protein phosphatase inhibitor-1 from rabbit skeletal muscle. Eur. J. Biochem. *126*, 235–246.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: John Wiley and Sons, Inc.).

Beals, C.R., Clipstone, N.A., Ho, S.N., and Crabtree, G.R. (1997). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes Dev. *11*, 824–834.

Bierer, B.E., Mattila, P.S., Standaert, R.F., Herzenberg, L.A., Burakoff, S.J., Crabtree, G.R. and Schreiber, S.L. (1990). Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. Proc. Natl. Acad. Sci. USA *87*, 9231–9235.

Cameron, A.M., Steiner, J.P., Roskams, A.J., Ali, S.M., Ronnett, G.V., and Snyder, S.H. (1995). Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates $Ca^{2+}$ flux. Cell *83*, 463–472.

Cardenas, M.E., Hemenway, C., Muir, R.S., Ye, R., Fiorentino, D., and Heitman, J. (1994). Immunoglobulins interact with calcineurin in the absence of exogenous immunosuppressive ligands. EMBO J. *13*, 5944–5957.

Clipstone, N.A., and Crabtree, G.R. (1992). Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation. Nature *357*, 695–697.

Coghlan, V.M., Perrino, B.A., Howard, M., Langeberg, L.K., Hicks, J.B., Gallatin, W.M., and Scott, J.D. (1995). Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science *267*, 108–111.

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. *58*, 453–508.

Crabtree, G.R., and Clipstone, N.A. (1994). Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu. Rev. Biochem. *63*, 1045–1083.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. *11*, 1475–1489.

Durfee, T., Becherer, K., Chen, P.-L., Yeh, W.-H., Yang, Y., Kilburn, A.E., Lee, W.-H., and Elledge, S.J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. *7*, 555–569.

Elledge, S.J., Mulligan, J.T., Ramer, S.W., Spottswood, M., and Davis, R.W. (1991). Lambda YES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and Escherichia coli mutations. Proc. Natl. Acad. Sci. USA *88*, 1731–1735.

Emmel, E.A., Verweij, C.L., Durand, D.B., Higgins, K.M., Lacy, E., and Crabtree, G.R. (1989). Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science *246*, 1617–1620.

Fields, S., and Song, O.-K. (1989). A novel genetic system to detect protein-protein interactions. Nature *340*, 245–246.

Flanagan, W.M., Corthesy, B., Bram, R.J., and Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature *352*, 803–807.

Frantz, B., Nordby, E.C., Bren, G., Steffan, N., Paya, C.V., Kincaid, R.L., Tocci, M.J., Okeefe, S.J., and Oneill, E.A. (1994). Calcineurin acts in synergy with PMA to inactivate IkB/MAD3, an inhibitor of NF-κB. EMBO J. *13*, 861–870.

Friedman, J., and Weissman, I. (1991). Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the abasence of CsA. Cell *66*, 799–806.

Fruman, D.A., Klee, C.B., Bierer, B.E., and Burakoff, S.J. (1992). Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc. Natl. Acad. Sci. USA *89*, 3686–3690.

Fruman, D.A., Pai, S.-Y., Burakoff, S.J., and Bierer, B.E. (1995). Characterization of a mutant calcineurin Aα gene expressed by EL4 lymphoma cells. Mol. Cell. Biol. *15*, 3857–3863.

Furukawa, T. (1995). Method for identifying CD45 PTPase substrate may be useful for other PTPases. J. NIH Res. *7*, 52–53.

Gardner, P. (1989). Calcium and T lymphocyte activation. Cell *59*, 15–20.

Handschumacher, R.E., Harding, M.W., Rice, J., and Drugge, R.J. (1984). Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science *226*, 544–547.

Harding, M.W., Galat, A., Uehling, D.E., and Schreiber, S.L. (1989). A receptor for the immuno-supressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. Nature *341*, 758–760.

Hashimoto, Y., Perrino, B.A., and Soderling, T.R. (1990). Identification of an autoinhibitory domain in calcineurin. J. Biol. Chem. *265*, 1924–1927.

Hsu, H.-L., Wadman, I., and Baer, R. (1994). Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. Proc. Natl. Acad. Sci. USA *91*, 3181–3185.

Hubbard, M.J., and Klee, C.B. (1989). Functional domain structure of calcineurin A: mapping by limited proteolysis. Biochemistry *28*, 1868–1874.

Jain, J., McCaffrey, P.G., Miner, Z., Kerppola, T.K., Lambert, J.N., Verdine, G.L., Curran, T., and Rao, A. (1993). The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature *365*, 352–355.

Jiang, H., Xiong, F., Kong, S., Ogawa, T., Kobayashi, M., and Liu, J.O. (1997). Distinct tissue and cellular distribution of two major isoforms of calcineurin. Mol. Immunol. *34*, 663–669.

Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H., and Imanaka, H. (1987). FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. J. Antibiot. *40*, 1249–1255.

Klauck, T.M., Faux, M.C., Labudda, K., Langeberg, L.K., Jaken, S., and Scott, J.D. (1996). Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science *271*, 1589–1592.

Klee, C.B., Draetta, G.F., and Hubbard, M.J. (1988). Calcineurin. Advances in Enzymology and Related Areas of Molecular Biology *61*, 149–209.

Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell *66*, 807–815.

Liu, J., Albers, M.W., Wandless, T.J., Luan, S., Alberg, D.G., Belshaw, P.J., Cohen, P., MacIntosh, C., and Klee, C.B. (1992). Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. Biochemistry *31*, 3896–3901.

Loh, C., Carew, J.A., Kim, J., Hogan, P.G., and Rao, A. (1996). T-cell receptor stimulation elicits an early phase of activation and a later phase of deactivation of the transcription factor NFAT1. Mol. Cell. Biol. *16*, 3945–3954.

Mondragon, A., Griffith, E.C., Sun, L., Xiong, F., Armstrong, C., and Liu, J.O. (1997). Overexpression and purification of human calcineurin a from Escherichia coli and assessment of catalytic functions of residues surrounding the binuclear metal center. Biochemistry *36*, 4934–4942.

Nelson, P.A., Akselband, Y., Kawamura, A., Su, M., Tung, R.D., Rich, D.H., Kishore, V., Rosborough, S.L., DeCenzo, M.T., and Livingston, D.J. (1993). Immunosuppressive activity of [$MeBm_2t$]$^1$, D-Diaminobutytyl-8-, and D-Diaminopropyl-8-Cyclosporin analogues correlates with inhibition of calcineurin phosphatase activity. J. Immunol. *150*, 2139–2147.

O'Keefe, S.J., Tamura, J., Kincaid, R.L., Tocci, M.J., and O'Neill, E.A. (1992). FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature *357*, 692–694.

Rechsteiner, M., and Rogers, S.W. (1996). PEST sequences and regulation by proteolysis. Trends Biochem. Sci. *21*, 267–271.

Shaw, K.T., Ho, A.M., Raghavan, A., Kim, J., Jain, J., Park, J., Sharma, S., Rao, A., and Hogan, P.G. (1995). Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. Proc. Natl. Acad. Sci. USA *92*, 11205–11209.

Sehgal., S.N., Baker, H., and Vezina, C. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. J. Antibiot. *28*, 727–742.

Shenolikar, S. (1994). Protein serine/threonine phosphatases: new avenues for cell regulation. Annu. Rev. Cell Biol. *10*, 55–86.

Shibasaki, F., Price, E.R., Milan, D., and McKeon, F. (1996). Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature *382*, 370–373.

Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S., and Sigal, N.H. (1989). A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature *341*, 755–757.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Nerian, Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. Cell *77*, 727–736.

Sugimoto, T., Stewart, S., and Guan, K.L. (1997). The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. J. Biol. Chem. *272*, 29415–29418.

Sun, H., Charles, C.H., Lau, L.F., and Tonks, N.K. (1993). MKP-1 (3CH134), an immediate early gene product, is a duel specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell *75*, 487–493.

Timmerman, L.A., Clipstone, N.A., Ho, S.N., Northrop, J.P., and Crabtree, G.R. (1996). Rapid shuttling of NF-AT in discrimination of $Ca^{2+}$ signals and immunosuppression. Nature *383*, 837–840.

Tordai, A., Sarkadi, B., Goroz, G., and Gardos, G. (1989). Inhibition of the CD3-mediated calcium signal by protein kinase. Immunol. Lett. *20*, 47–52.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grandperret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., and Loriolle, F., (1991). The bisindolylmaleimide GF 109023X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. *266*, 15771–15781.

Wesselborg, S., Fruman, D.A., Sagoo, J.K., Bierer, B.E., and Burakoff, S.J. (1996). Identification of a physical interaction between calcineurin and nuclear factor of activated T cells. J. Biol. Chem. *271*, 1274–1277.